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Award Number: W81XWH-04-1-0320

TITLE: INVESTIGATION OF NOVEL MOLECULAR TARGETS FOR PLECKSTRIN HOMOLOGY (PH) DOMAINS FOUND IN ONCOGENES IMPLICATED IN BREAST CANCER

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REPORT DATE: March 2007

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-03-2007	Annual Summary	23 Feb 04 – 22 Feb 07

4. TITLE AND SUBTITLE
INVESTIGATION OF NOVEL MOLECULAR TARGETS FOR PLECKSTRIN HOMOLOGY (PH) DOMAINS FOUND IN ONCOGENES IMPLICATED IN BREAST CANCER

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0320

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Pennsylvania
Philadelphia PA 19104-6205

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT: Plecktrin Homology (PH) domains are commonly thought of as membrane-targeting modules involved in signaling pathways that bind phosphoinositides (PPIns) with high affinity and specificity. In a recent study of S. cerevisiae, however, the vast majority demonstrated little affinity or specificity for PPIns (Yu et al, 2004). I show comparable results for my selected human PH domains, with only one that is high affinity and PPIns-specific, while the remainder are low to moderate affinity and promiscuous for PPIns. I outline two instances where multipoint contacts (protein-protein and protein-phosphoinositide interactions) may account for specific membrane targeting observed in vivo. First, SH3BP-2 PH was identifed as highly specific for the membrane lipid PtdIns(3,4)P2, and targets the host adaptor protein to the membrane (while its SH2 and PR domains bind proteins). Second, FAPP1 and OSBP PH domains possess comparable affinities for Golgi- and plama membrane-enriched PPIns in vitro, although they both localize to the Golgi, and not the plasma membrane in vivo, a likely result of a direct interaction with the Golgi GTPase Arf1 (Godi et al, 2004). I have solved the structure of a member of this PH domain class in complex with PPIns, and suggest reasons for its unique PPIns binding properties. Finally, the drug perifosine was found to selectively target Akt1/PKBα PH in vitro, albeit with a relatively low binding affinity.

15. SUBJECT TERMS
Oncogene, Signaling Domain, Interaction, Membrane Targeting

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	21	**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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Introduction

The primary goal of my studies has been to understand how PH domain recognition of phospholipids and of other proteins contributes to signaling by the wide array of molecules that contain PH domains – the 11th most common domain in the human genome, and one that is present in many proteins implicated in breast cancer (Cesareni *et al*, 2004; Schmidt and Hall, 2002). Elucidation of the nature of these PH domain interactions (and how they are regulated) in sufficient detail may suggest approaches for inhibiting these interactions pharmacologically. This report will enumerate the advances I have made towards reaching these goals. In addition to the proposed *in vitro* phospholipid binding studies and *in vivo* cellular localization studies, I have also characterized a specific PH domain-alkylphospholipid drug interaction *in vitro*, and have completed the structural determination of a member of a poorly characterized class of PH domains bound to phospholipid. At least two members of this class of PH domains (FAPP2 and OSBP2) have been associated with breast cancer development or progression (Fournier *et al*, 1999; Scanlan *et al*, 2001).

Following the format of my proposed Statement of Work, my progress towards this aim is summarized as follows:

Task 1 Investigate affinity and specificity of phosphoinositide binding to isolated human PH domains

I have analyzed the phosphoinositide binding specificity of 21 of the 66 phylogenetic representative human PH domains proposed for study with lipid overlay assays (dot blot) and SPR lipid-binding assays, as previously described (Kavran *et al*, 1998; Yu *et al*, 2004). An extensive literature search confirmed that another 8 PH domains that have already bee characterized for phosphoinositide binding *in vitro* (Yu *et a*l, 2006; Rajala *et a*l, 2005; Yan *et al*, 2005; Skowronek *et al*, 2004; Saxena *et al*, 2002; Dowler *et al*, 2000; Fleming *et al*, 2000). As expected from our prior analysis of yeast PH domains, the majority of the human PH domains selected bind phosphoinositides promiscuously and with only low affinity (compare **Tables 1 & 2**). Six of the human PH domains tested possessed high affinity and specificity for phosphoinositides (either for PtdIns(4,5)P_2, PtdIns(3,4,5)P_3, or PtdIns(3,4)P_2/PtdIns(3,4,5)P_3; see **Figure 1**), and only one yeast PH domain. The two human PH domains of OSBP1 and FAPP1 represent a separate group of domains that are promiscuous in their phosphoinositide binding, yet have moderate-to-high affinity for these phospholipids when assessed using surface plasmon resonance studies. The corresponding yeast orthologs of mammalian OSBP are Osh1p and Osh2p (Lehto *et al*, 2001; Yu *et al*, 2004).

The OSBP/FAPP/Osh PH domain family has been at times both poorly characterized and mischaracterized *in vivo* and *in vitro*. Several of these PH domains were initially identified as PtdIns4*P*-specific *in vitro*, and, since that time, have been consistently misused as markers of cellular PtdIns4*P* *in vivo* (Várnai and Balla, 1998; Dowler *et al*, 2000). In contrast to reported PtdIns4*P* specificity, I have determined that the PH domains of OSBP1 and FAPP1 display *in vitro* binding affinities that are comparable between PtdIns(4)*P* (K_d 3.5 and 21 μM, respectively) and PtdIns(4,5)P_2 (K_d 3.3 and 17 μM, respectively), as measured by SPR lipid-binding assays (**Figure 2**). These results are consistent with those reported for the fusion PH domain constructs in an earlier study (Levine & Munro, 2002). When the assay was expanded to include other

phosphoinositides (clear area of **Table 4**), OSBP1 was found to have comparable *in vitro* binding affinities for all phosphoinositides tested (Kd 3.2-3.8 μM), except for the two-fold weaker affinity of PtdIns3*P* (apparent Kd 6.5 μM) and no binding for PtdIns5*P*. These data mirror published *in vitro* binding affinities of the yeast ortholog Osh1p PH, and sharply contrast with the high affinity and PtdIns(4,5)P_2 specificity of PLCδ PH (shaded area of **Table 4**). Interestingly, FAPP1 PH appears to have an intermediate specificity for phosphoinositides; in addition to the comparable binding affinities for PtdIns(4)*P* and PtdIns(4,5)P_2 (K_d 21 and 17 μM, respectively), it has a two-fold weaker affinity for PtdIns(3,5)P_2 (K_d 31.4 μM), but displays no binding for other phosphoinositides. The implications of these data will be discussed later.

Another outcome of these studies was the identification of a human PH domain with unexpected phosphoinositide-binding properties. As shown in **Figure 3**, the PH domain of SH3BP-2 (3BP-2) is highly specific for PI(3,4)P_2, but with only moderate affinity, unlike other high affinity, 3-phosphoinositide binding PH domains (like Akt1/PKBα PH). The K_d is 0.3-0.5 μM for the dimeric GST-PH fusion construct (and an estimated ten-fold weaker for monomer (Kavran *et al*, 1998)). SH3BP-2 is an adaptor protein with a versatile role in a variety of cell types (**Figure**). It is in the same phylogenetic PH domain class as that of DAPP1 PH (a PtdIns(3,4)P_2/PtdIns(3,4,5)P_3-specific, high affinity PH domain), and, like DAPP1 PH, is translocated to the plasma membrane upon PI3K activation (http://www.signaling-gateway.org/). These results are consistent with the protein's PI3K-dependent roles as a positive regulator of IL-2 gene induction in T cells (Deckert *et al*, 1998), NK cell-mediated cytotoxicity (Jevremovic *et al*, 2001), and FcεRI-induced degranulation and signal amplification in mast cells (Sada *et al*, 2002).

Task 2 Determine the subcellular localization of the PH domains.

Since PH domains are most commonly thought of as membrane-targeting modules (a function of their phospholipid binding), one might expect that PH domains with high, but not low, affinities for phosphoinositides should be membrane-localized *in vivo*. I strongly suspected this would not be true universally for all human PH domains, as several low affinity PH domains showed a surprising degree of localization to various cellular membranes in a previous yeast genomewide study (Yu *et al*, 2004). In my original proposal, I proposed an *in vivo* fluorescence localization study of the representative human GFP-PH domain fusion constructs. By 2005, the Alliance for Cell Signaling (AfCS) laboratory at Stanford had completed an comparable study of mouse PH domain localization in two separate mouse cell lines (72 in WEHI-231 cells and 148 in RAW 264.7 cells) (http://www.signaling-gateway.org/). This study provided *in vivo* localization data covering 45 of the 66 human PH domains phylogenetic classes listed in our original proposal (including 18 of the 21 PH domains that I had tested for phosphoinositide binding)(**Tables 2 & 3**). Therefore, I elected not to reproduce the *in vivo* studies already completed by the AfCS study. The overall distribution of cytosolic versus membrane- or puncta-localized PH domains is comparable to that of our yeast PH domain study. Furthermore, as with the yeast study, several human PH domains with low affinities for phosphoinositides *in vitro* unpredictably demonstrated plasma membrane or punctate localization *in vivo*.

In order to determine the cellular localization of OSBP1 PH and FAPP1 PH *in vivo*, I transfected GFP fusion constructs into live NIH-3T3 and HeLa cells. The fluorescence localization in these cells was punctate in nature (**Figure 4**), consistent with Golgi membrane

localization, as found in previous studies (Levine & Munro, 2002; Godi *et al*, 2004; Balla *et al*, 2005). The *in vivo* data is also consistent with the reported roles of this class of PH domains in coordinating budding and fission events at the Golgi for the generation of cargo transporters targeted for fusion with the plasma membrane (Itoh & De Camilli, 2004; Roth 2004). The data are seemingly at odds, however, with the aforementioned *in vitro* data demonstrating the promiscuity (and relatively weak affinity, in the case of FAPP1 PH) for phosphoinositides. Specifically, the observation that OSBP/FAPP/Osh1 PH family members target to the PtdIns4*P*–rich Golgi rather than the PtdIns(4,5)P_2-rich plasma membrane *in vivo*, despite no apparent difference in *in vitro* binding affinities for their respective phosphoinositide, is unexpected. This is the subject of the subsequent task.

Task 3 Screen for putative interacting proteins of PH domains

Since phosphoinositide binding alone cannot account for the specific subcellular localization of several PH domains – particularly in the case of the OSBP/FAPP/Osh1 PH family – it has been suggested that other targets, particularly proteins, may help define their localization. Levine and Munro observed that Golgi targeting of the OSBP1 PH domain requires both PtdIns4*P* and a second PI4K-independent determinant, which they suggested from genetic studies might be Arf1, a Golgi small GTPase (Levine & Munro, 2002). More recently, the PH domains of both OSBP1 and FAPP1 were found to specifically and directly interact with Arf1 *in vitro* (Godi *et al*, 2004).

I have utilized purified myristoylated Arf1 (DNA construct provided by Paul Randazzo (Manser & Leung, 2002)) to test its reported interaction with GST-tagged FAPP1 PH and OSBP PH using GST-pulldown assays (similar to the one published in Godi *et al*, 2004). Results confirm what appears to be a relatively weak and non-robust interaction *in vitro* (**Figure 5**). Although the PH domain literature is replete with instances of *in vitro* pulldowns of putative protein partners, there are inherent limitations in relying on this approach to prove direct, biologically significant PH domain-protein interactions *in vivo*. My previously stated goal was to quantitatively measure the binding affinity of this putative PH domain-Arf1 interaction.

There appears to be some consensus that both Arf1 and PtdIns4*P* are both necessary to provide a sufficiently strong interaction with the PH domains *in vivo* (Itoh & De Camilli, 2004; Godi *et al*, 2004; Levine & Munro, 2002). The challenge is to present both the myristoylated Arf1 and PtdIns(4)*P* in sufficient proximity to one another to allow simultaneous interactions with the PH domains, and to maintain a cellular membrane-like structure that would convincingly demonstrate the likelihood of such an interaction occurring *in vivo*. To accomplish this task, I plan to generate combined myrArf1/phosphoinositide (PtdIns4*P*:PC) vesicles to quantitatively measure the binding affinity of the interaction using SPR assays. I have found one instance in the literature where myrArf1 was successfully incorporated into lipid vesicles, and plan to broadly follow its protocol (Randazzo 1997). This project is still in progress.

Task 4: Structure Determination of Osh1p PH domains

One of the primary motivations of this study is to structurally characterize PH domain-phosphoinositide ligand interactions in detail. In addition to the seeming disconnect between *in*

vitro binding affinity data and *in vivo* cellular localization data (which may be at least partly explained by the existence of the Golgi-based protein partner Arf1), a basic issue concerning the similar *in vitro* binding affinities between PtdIns(4)*P* and PtdIns(4,5)P_2 for Osh1p/OSBP/FAPP1 PH still remains unanswered. All previously published PH domain structures (liganded or liganded) interact with two adjascent ligand determinants (whether the 3-*P* and the 4-*P* of DAPP1 PH/Ins(1,3,4,5)P4 complex, the 4-*P* and the 5-*P* of PLCδ PH/Ins(1,4,5)P3 complex, or simply two free phosphate groups of unliganded DAPP1 PH (DiNitto *et al*, 2003)). As phosphate groups on the inositol head group are the major PH domain determinants, how (and why) would a PH domain recognize a monophosphoinositide and diphosphoinositide with similar binding affinities? What are the structural determinants that account for PH domain promiscuity? I am addressing this question using two approaches.

Upon performing a gel filtration assay, I observed that while some [^{3}H]-Ins(1,4,5)P_3 (PtdIns(4,5)P_2 headgroup) coeluted with GST-PLCδ PH, no [^{3}H]-Ins(1,4,5)P_3 was detected coeluting with the GST-PH domains of Osh1p, OSBP, or FAPP1 (**data not shown**). These results suggest that the Osh1p/OSBP/FAPP1 PH domain family bind Ins(1,4,5)P_3 weakly (<1 μM, the approximate detection limit of this assay) or not at all. To distinguish between these possibilities and further identify whether the determinants are the glycerol moiety or nearby acyl chains of the phosphoinositide, I performed SPR lipid competition studies, preincubating inositol headgroups / phosphoinositides of different lengths with the OSBP PH before applying it to lipid (3% PtdIns(4)*P* and PtdIns(4,5)P_2 in PC background) surfaces (**Figure 6A**). I also tested InsP_6, (which unexpectedly coeluted with the PH domains in the gel filtration assay), and Ins(1,3,4,5,6)P_5 as a control (to determine whether InsP_6 binding is InsP_6-specific or nonspecific due to high negative charge density (Shears 2002)). The results (**Figure 6B**) were consistent with gel filtration data:

1) Ins(1,4,5)P_3 did not compete OSBP PH off PtdIns(4)*P* or PtdIns(4,5)P_2 surfaces, suggesting headgroup contacts alone are weak relative to the entire phosphoinositide;

2) both InsP_6 and Ins(1,3,4,5,6)P_5 effectively competed OSBP PH off PtdIns(4)*P* and PtdIns(4,5)P_2 surfaces, suggesting the importance of nonspecific electrostatic interactions;

3) all inositol phosphates and phosphoinositides (except C4-PtdIns(4)*P*) competed PLCδ PH off PtdIns(4,5)P_2 surface as expected, since PLCδ PH strongly interacts with headgroup (Note: PLCδ PH does not interact with PtdIns(4)*P* surface).

Additionally, **4**) short-chain phosphoinositides C4-PtdIns(4)*P* and C4-PtdIns(4,5)P_2 did noticeably compete OSBP PH off PtdIns(4)*P* and PtdIns(4,5)P_2 surfaces, suggesting a rather modest contribution from the glycerol moiety and/or acyl side chains.

The data suggest that membrane surface interactions and/or insertion into the membrane may contribute quite substantially to the promiscuity observed in the Osh1p/OSBP/FAPP1 PH domain family.

To identify the specific contribution of the ligand determinants to PH domain binding, I proposed to determine the structure of the PH domain complexed with phosphoinositides. I have prepared crystals of monomeric His-tagged Osh1 PH complexed with the soluble, short chain (C4) derivatives of both PtdIns(4)*P* and PtdIns(4,5)P_2 (**Figure 7A**). I have collected a full structure data set of the PtdIns(4,5)P_2– and PtdIns(4)*P*–complexed Osh1 crystals on-site, and have identified a molecular replacement solution working with DAPP1 PH. While I am still in the process of obtaining higher resolution data, some interesting details are emerging. The phosphate "footprints" in the Osh1 PH/PtdIns(4,5)P_2 electron density map are not in the position

that one would expect for two adjascent phosphates, but rather two non-adjascent ones, a result never observed for PH domains up to now (**Figure 7B**). Additionally, there is only one prominent phosphate "footprint" in the Osh1 PH/PtdIns(4)*P* electron density map. In both instances, the phosphoinositide (and the critical β1-β2 loop) are positioned further outside the Osh1 PH lipid binding site then that observed for other PH-PPIns complexes, an observation that explains why the Osh1p/OSBP/FAPP family of PH domains bind more weaky and promiscuously to phosphoinositides then members the high affinity and specific PH domain class (eg.- PLCδ PH; see **Table 4**). Interactions with other phosphoinositide determinants (phosphate or acyl chain) await a higher resolution structure, to be completed by next month.

PH domains as drug targets in cancer therapy

Our original view of PH domains suggested that phosphoinositide-binding PH domains themselves should make poor targets for pharmacological intervention, since most phosphoinositide-recognition events are essentially the same, and, moreover, drugs likely to target PH domains are very highly charged, which leads to delivery problems. Recently, I was presented with a unique opportunity to test the PH domain-binding properties of perifosine, a C_{18}-alkylphospholipid drug that has completed phase I trials (Van Ummersen *et al*, 2004; Crul *et al*, 2004) as an anti-cancer agent. Earlier immunoprecipitation studies suggested that perifosine specifically inhibits Ser/Thr phosphorylation and kinase activation of Akt1/PKBα *in vivo* and *in vitro* (Kondapaka *et al*, 2003). Myristoylated Akt1/PKBα, which is targeted directly to the plasma membrane in a PH domain-independent manner, is unaffected by perifosine treatment. It was therefore hypothesized that perifosine might act by directly interfering with the phosphoinositide binding (or other membrane-targeting interaction) of the Akt1/PKBα PH domain. I have recently completed a series of SPR binding studies suggesting that perifosine specifically competes with phosphoinositides for binding to the PH domain of Akt1/PKBα (EC_{50} 26 μM), while it competes substantially less for binding to the PH domain of PLCδ, and not at all for the PH domains of DAPP1 (**Figure 8**) and FAPP1 (data not shown). These studies indicate that perifosine may bind directly to the phosphoinositide-binding site of the Akt1/PKBα PH domain.

Key Research Accomplishments

- GST-PH domain fusion constructs (21 of the 66 representative PH domains) have been tested for their phosphoinositide affinity and specificity *in vitro* by dot blot and SPR(BIAcore) assays. The overall distribution of high, moderate, and low affinity PH domains in the human proteome appears to be comparable with that of yeast.
- GFP-PH domain fusion constructs of several moderate affinity and promiscuous PH domains were tested for *in* vivo localization in MDA-MB-468 and NIH 3T3 cells, demonstrating punctate localization.
- The binding affinities of monomeric OSBP1 PH and FAPP1 PH for all phosphoinositides using SPR assays were tested demonstrating the promiscuity of OSBP1 PH and, to a lesser extent, FAPP1.
- Differences between phosphoinositide binding *in vitro* between OSBP1 PH and PLCδ PH were observed in gel filtration and SPR competition studies, suggesting that the inositol head group or

nearby acyl chains cannot fully account for the full extent of PH domain-phospholipid surface interaction of this class of PH domains.

- Weak myrArf1 interactions with OSBP PH and FAPP1 PH were demonstrated *in vitro* using GST pulldown assays .
- Completed structure determination using molecular replacement solution for Osh1p PH in complex with both PtdIns4*P* and PtdIns(4,5)P_2.
- A unique PH domain (SH3BP-2) with moderate affinity, yet high specificity for PI(3,4)P_2 has been characterized.
- An alkylphospholipid drug has been identified as highly specific for the PH domain of Akt1/PKBα to the exclusion of several other related PH domains by SPR competition assays, despite its relatively weak affinity as determined by ITC.

Reportable Outcomes

- Publications include contributing authorship in peer-reviewed journal (Yu *et al*, 2004), book chapter review of PH domains (Cesarini *et al* (ed.), 2004), and review article (in press).
- Abstracts and posters for Era of Hope Meeting and Annual Departmental Retreats.
- GST- and GFP-PH domain fusion constructs for many of the representative PH domains that had been originally proposed have been cloned.
- Complete phylogenetic tree (dendrogram) of the entire human and yeast PH domain proteome.
- Complete phylogenetic tree (dendrogram) of other protein-binding domains of the human proteome including PTB, EVH/WH1, and RanBD.
- Molecular coordinate model for Osh1p PH complexed with both PtdIns4*P* and PtdIns(4,5)P_2 at 2Å resolution.

Conclusion

The key question underlying all of my research accomplishments to date is: How do nonspecific and specific component interactions cooperate to drive selective membrane targeting? I have demonstrated that membrane targeting by the PH domain requires multipoint contacts, which may include both protein-protein and protein-lipid interactions (**Figure 9**). In the case of OSBP1 and FAPP1 (**Figure 9A**), it appears that the PH domain must simultaneously interact with both Arf1 and phosphoinositide to target to the Golgi (although I have not yet quantifted this interaction). In the case of SH3BP-2 (**Figure 9B**), the SH2 and PR domains both cooperate with the PtdIns(3,4)P_2-specific PH domain (which I have characterized) to drive plasma membrane targeting upon receptor activation. In both instances, these relatively weak affinity, high specificity protein-protein interactions are supplemented by of moderate affinity protein-phosphoinositide interactions.

I have also demonstrated that, with regard to phosphoinositide recognition of PH domains, not all phosphoinositide interactions among PH domains are equivalent. I have described a class of PH domains which bind monophosphoinositides and diphosphoinositides with comparable affinity *in vitro,* although they target to specifically to the Golgi *in vivo* (**Figure 9C**). At least part of this promiscuity can be explained by membrane surface interactions or insertion of the PH domain. The structural model I have at this stage suggests a unique arrangement of PH domain interactions with nonadjascent phosphates on the phosphoinositide,

an observation that is unique among PH domain structures defined to date. Additionally, I have characterized an alkylphospholipid drug (perifosine) which appears to have high specificity, but low affinity, for Akt1/PKBα PH, a PH domain with high affinity and specificity for the 3'-phosphoinositides PtdIns(3,4)P_2.and PtdIns(3,4,5)P_3 (**Figure 9D**).

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Supporting Data

Table 1 Yeast PH Domains

Num1p		
Cla4p	Boi1p	
Skm1p	**Boi2p**	
Osh1p	***Osh2p***	
Ask10p	Syt1p	Caf120p
Bem2p	Ugt51p	Gcs1p
Cdc24p	Ybl060p	Opy1Ap
Opy1Bp	Yhr131p	Tus1p
Osh3p	**Yil105cp**	Yhr155wp
Sip3p	Ylr187wBp	Ynl144p
Spo14p	**Ynl047cp**	Ypr091cp
Spo71Bp	Ypr115wp	Spo71Ap
Stt4p	Bem3p	Ylr187wAp

Table 2 Human PH Domains

PLCδ_1		
Tiam1-Ct	DAPP1 / PHISH	**Gab2**
Cytohesin 2 / ARNO	*TAPP1-Ct*	
*FAPP1**	*OSBP1**	*Trio-Nt*
Vav1	AP20 / LL5β	PLD1
Dbl	FGD1-Nt	**Dok1**
Sos1	*FGD1-Ct*	Pleckstrin-Nt
IRS1	**DAGKδ**	Dynamin 1
PEPP1	Syntrophin-3	Grb14
TAPP1-Nt	Tiam1-Nt	Ipl
βARK1	KIAA0053, RhoGAP25	Myosin X-Nt

Table 3 Mouse PH Domains (Localization)

PM localization	**Cytosolic/Nuclear**	**Nuclear**	**Puncta**
Lbc / Ht31	Tiam1	JBP / TNFidp	Kif1a
Gab2	Grb14		TAPP2-Nt
Phafin 1	Net1		
Spectrin β2	PKCμ / PKD		
ORP3	Oligophrenin 1		
CNK2	Centaurin δ_2 / ARAP1		
	GNRP-Nt, Ct		
	Trad / Duet		
	NGEF		
	Rasal (RasGAP)		
	CAPS		
	Tec		
	SH2β		
	TAPP2-Ct		

Table 1 Yeast PH domain affinity *in vitro* and localization *in vivo*

Color: High affinity and PI(4,5)P_2-specific in red, High affinity and PI(3,4)P_2/PI(3,4,5)P_3-specific in green, Moderate affinity and promiscuous in purple, Low affinity and promiscuous in blue. Font: Cytosolic and nuclear localization is regular, Plasma membrane localization is **bold**, Punctate localization is *italicized* (see Yu *et al*, 2004 for details).

Table 2 Human PH domain affinity *in vitro* and localization *in vivo*

See above for color and font key (Sources for affinity and localization include Kavran *et al*, 1998; Ferguson *et al*, 2000; Snyder *et al*, 2001, and unpublished data). Localization data from mouse was used (See WEHI-231 and RAW 264.7 Image Data in http://www.signaling-gateway.org for details). * While not on the list of 66 representative PH domains, the PH domains of OSBP1 and FAPP1 are related to FAPP2. In particular, the PH domains of FAPP1 and FAPP2 have similar phosphoinositide- and Arf1-binding properties *in vivo* and *in vitro* (Godi *et al*, 2004).

Table 3 Mouse PH domain localization *in vivo*

Font: Black for PH domains represented in original proposal, Violet for alternate PH domains in the same phylogenetic class. Note that each PH domain listed represents a single phylogenetic class. (See WEHI-231 and RAW 264.7 Image Data in http://www.signaling-gateway.org for details).

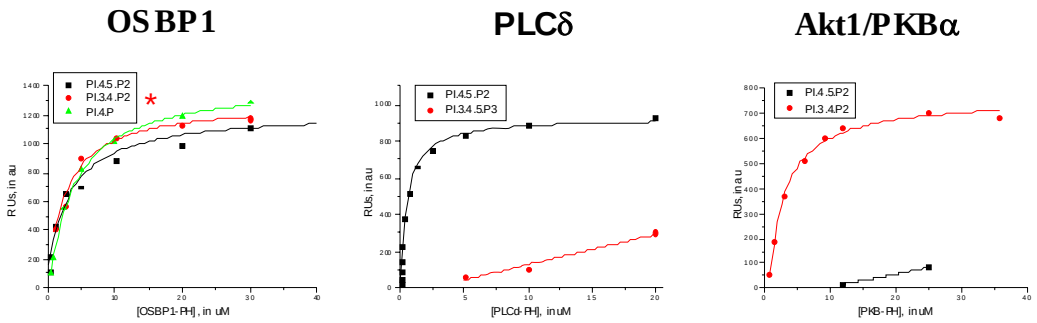
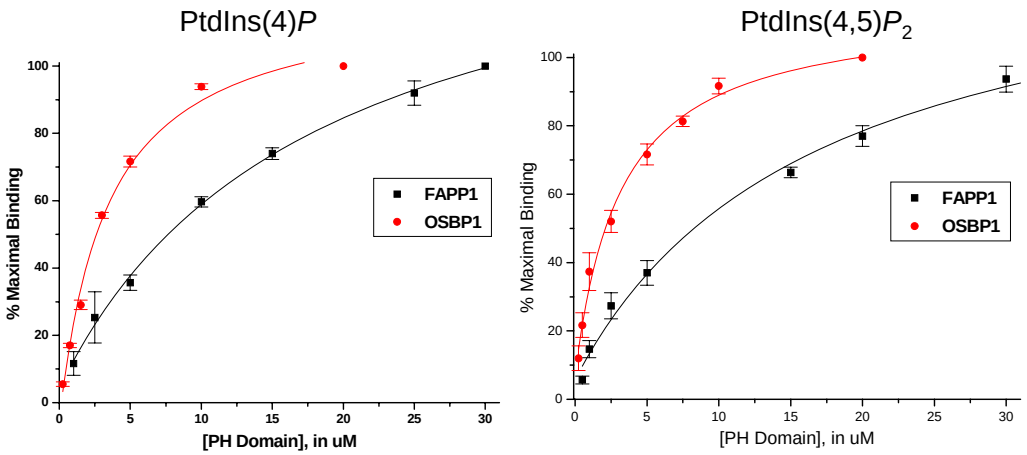


Figure 1
SPR binding data for OSBP PH suggest promiscuity for phosphoinositides.
SPR binding assay protocol detailed in Yu *et al*, 2004.
BIAcore response is based on refractive index changes that accompany protein binding to a lipid-coated chip surface (PtdIns(4,5)P_2, PtdIns(3,4)P_2, PtdIns4P). The apparent Kd is calculated from repeated iterations of Y=(Rmax*((1/Kd)*X)/(1+((1/Kd)*X)))+cf, where Rmax is the maximal response and cf is the correction factor.



PH Domain	PtdIns3*P*	PtdIns4*P*	PtdIns5*P*	PtdIns(3,4)*P2*	PtdIns(3,5)*P2*	PtdIns(4,5)*P2*	PtdIns(3,4,5)*P3*
OSBP	6.5 ± 1.3	3.4 ± 0.7	NB	3.8 ± 1.1	3.4 ± 0.4	3.2 ± 0.6	3.6 ± 1.4
FAPP1	NB	17.8 ± 2.4	NB	NB	31.4 ± 5.5	16.7 ± 3.6	NB
PLCd	> 100 μM	131 ± 19	NB	NB	76.0 ± 4.7	0.68 ± 0.28	NB
Osh1	6.2 ± 1.3	2.8 ± 0.8	------	------	3.5 ± 0.8	3.0 ± 1.0	------
Osh2	1.5 ± 0.2	1.3 ± 0.2	------	------	1.0 μM	1.1 ± 0.3	------

Figure 2 SPR binding curves for OSBP PH and FAPP1 PH for PtdIns(4,5)P_2 and PtdIns4*P* surfaces. SPR binding assay protocol detailed in Yu *et al*, 2004.
BIAcore response is based on refractive index changes that accompany protein binding to a lipid-coated chip surface. The apparent Kd is calculated from repeated iterations of Y=(Rmax*((1/Kd)*X)/(1+((1/Kd)*X)))+cf, where Rmax is the maximal response and cf is the correction factor.

Table 4 SPR binding data for OSBP PH and FAPP1 PH for all phosphoinositides
OSBP PH and FAPP1 PH data showed alongsude published data of PLCδ PH, Osh1 PH, and Osh2 PH (Yu *et al*, 2004). Note that no positive controls have been identified for PtdIns5*P*.

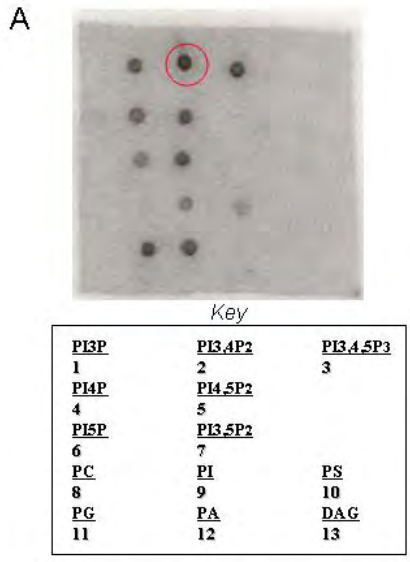


Fig.3A SH3BP-2 PH domain is a moderate affinity, PI(3,4)P2-specific PH domain.
Protein-Lipid Overlay Assay protocol detailed in Yu *et al*, 2004
The phospholipids are spotted at the positions indicated on the key. The intensity of the spots are indicative of radiolabeled protein bound to lipid. GST-PH domain fusion of SH3BP-2 shows selectivity for PI(3,4)P2.

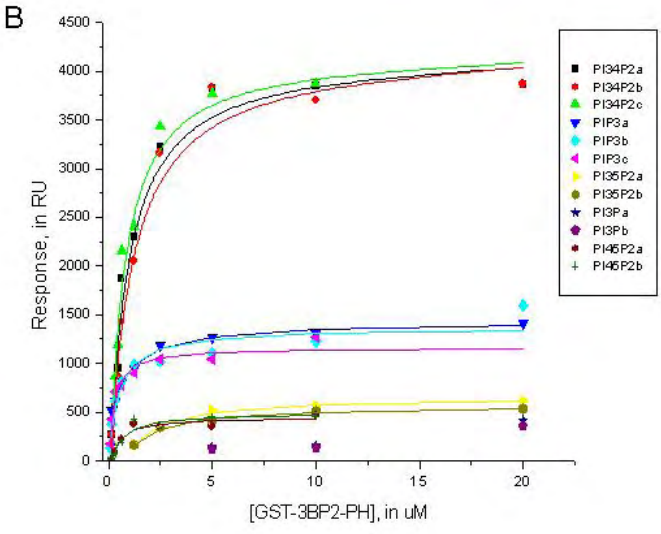


Fig.3B SH3BP-2 PH domain is a moderate affinity, PI(3,4)P2-specific PH domain
SPR Binding Assay protocol detailed in Yu *et al*, 2004
BIAcore response is based on refractive index changes that accompany protein binding to a lipid-coated chip surface. The apparent Kd is calculated from repeated iterations of: Y=(Rmax*((1/Kd)*X)/(1+((1/Kd)*X)))+ff, where Rmax is the maximal response and cf is the correction factor.

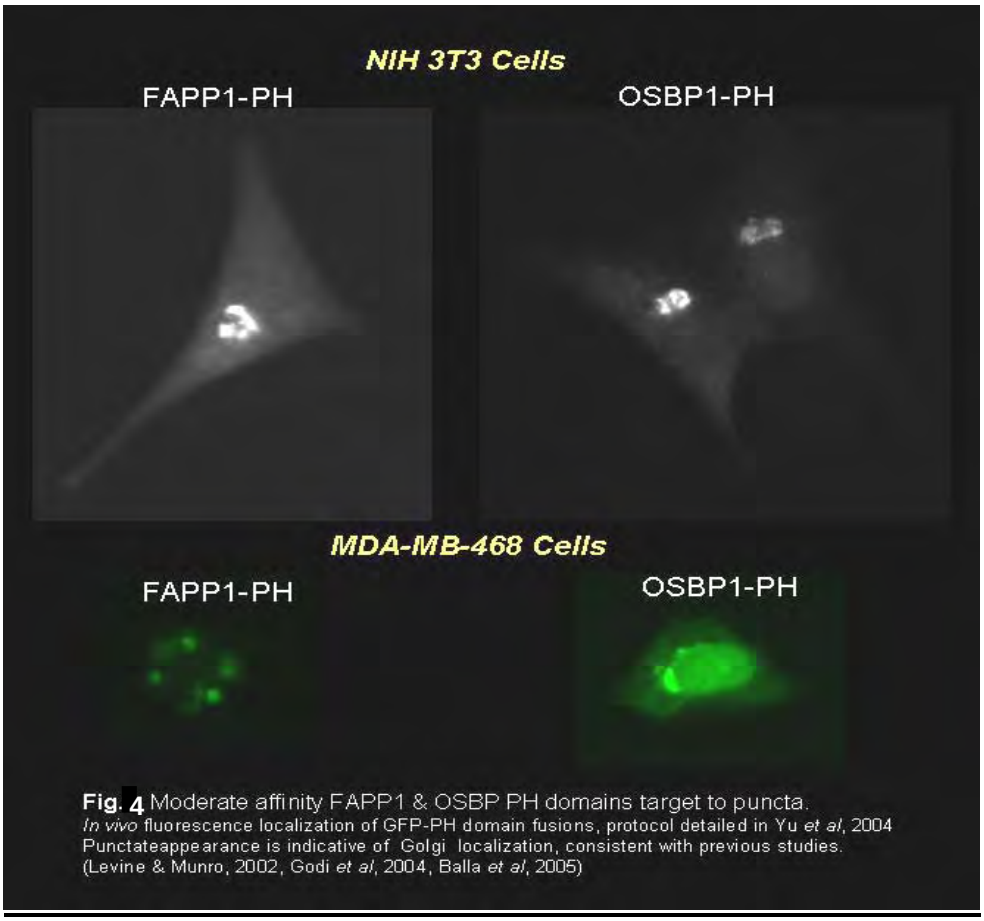


Fig. 4 Moderate affinity FAPP1 & OSBP PH domains target to puncta.
In vivo fluorescence localization of GFP-PH domain fusions, protocol detailed in Yu *et al*, 2004
Punctateappearance is indicative of Golgi localization, consistent with previous studies.
(Levine & Munro, 2002, Godi *et al*, 2004, Balla *et al*, 2005)

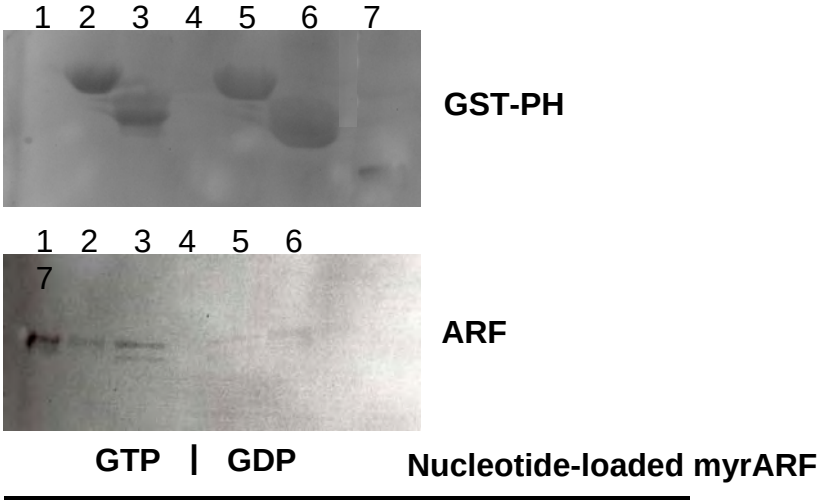


Figure 5 PH domain-myrARF1 coprecipitation.

GST pulldown data suggest weak myrArf1 interactions with OSBP and FAPP1 PH.

Recombinant myristoylated Arf1 was expressed and purified as previously described (Cesareni *et al*, 2004). GST-OSBP-PH and GST-FAPP1-PH were expressed and purified as previous GST-tagged proteins, with the exception that they were retained immobilized, and not eluted, on the glutathione-Sepharose beads (Lemmon *et al*, 1995; Yu *et al*, 2004). myrArf1 was loaded with 100 μM GTP-γS or GDP by a 1 hr incubation at 32^0C in HEPES loading buffer, followed by a 1 hr. incubation with GST-PH domain immobilized on glutathione-Sepharose beads at RT, as described previously (Godi *et al*, 2004). Beads were collected by low-speed centrifugation, washes 3X, and resuspended in 3X sample buffer. Sample was boiled for 5 min at 95^0C, and run on a 15% SDS-PAGE. Proteins were transferred to nitrocellulose paper by Western blot, blocked for 10 min in Blotto buffer with 5% dry milk, and incubated with **A)** mouse anti-GST antibody (1:1000, QIAgen), or **B)** goat anti-Arf1 antibody (1:1000, Santa Cruz Biotech) in 1X PBS O/N at 4^0C. The blot was washed 3X with 1X PBS, followed by a 1 hr incubation at 4^0C with secondary antibody (rabbit anti-mouse (Amersham) and donkey anti-goat (Santa Cruz Biotech), respectively), and washed again 3X. Finally, the blot was developed with ECL reagents, as per the manufacturer's (Amersham) instructions.

Lanes 1 myrARF1
Lanes 2, 5 myrARF1+GST-OSBP1 PH
Lanes 3, 6 myrARF1+GST-FAPP1 PH
Lanes 7 GST alone

Possible outcomes after testing phospholipids

IP3	+	-	-	-	-	-
gIP3	+	+	-	-	-	-
di-C4-PIP2	+	+	+	-	-	-
di-C8-PIP2	+	+	+	+	-	-
PIP2	+	+	+	+	+	-
Surface	+	+	+	+	+	+

PLCδ would be displaced by any of these constructs.

Binds head group

Binds glycerol group

Binds acyl group

Try L-B (membrane insertion)

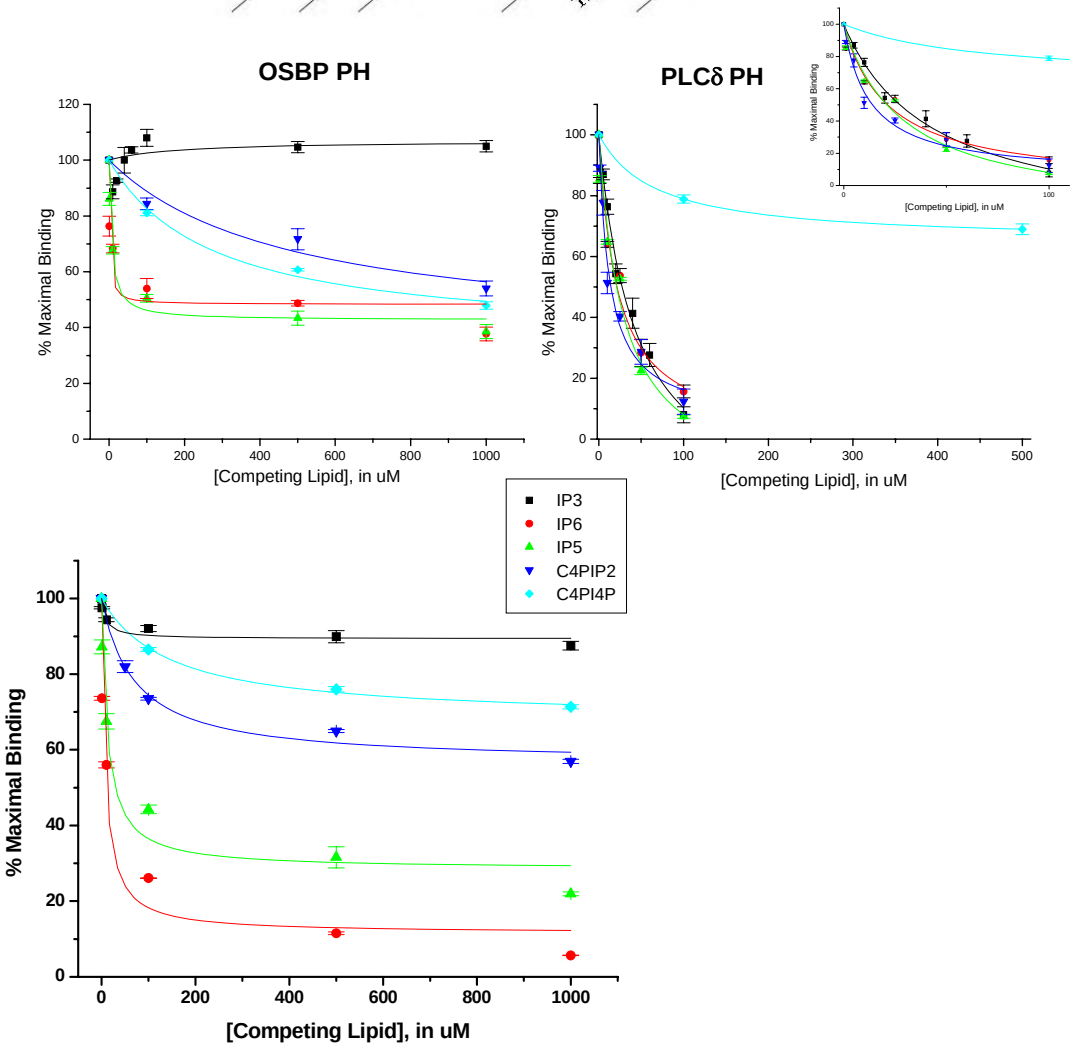


Table 5: Original scheme for SPR competition studies. gIP3 and surface interaction testing were unavailable**.**

Figure 6: SPR competition studies of OSBP PH and PLCδ PH on a PtdIns(4,5)P_2 (*top panels*) and OSBP PH on PtdIns4P (*bottom panel*) surfaces, with indicated InsP/sc PPIns preincubated with the PH domain before flowing over a lipid surface. All datapoints (Rmax) are normalized against non-precomplexed OSBP standard and fit to the curve: $\%_{comp}=\{\%_0*[IC_{50}/(C+IC_{50})]\}+\%_\infty$ where $\%_{comp}$ is RUs at defined concentration, C; $\%_0$ is the Bmax (where inhibition plateaus); IC_{50} is the concentration of competing lipid at 50% inhibition; $\%_\infty$ is the coefficient of variance (CV).

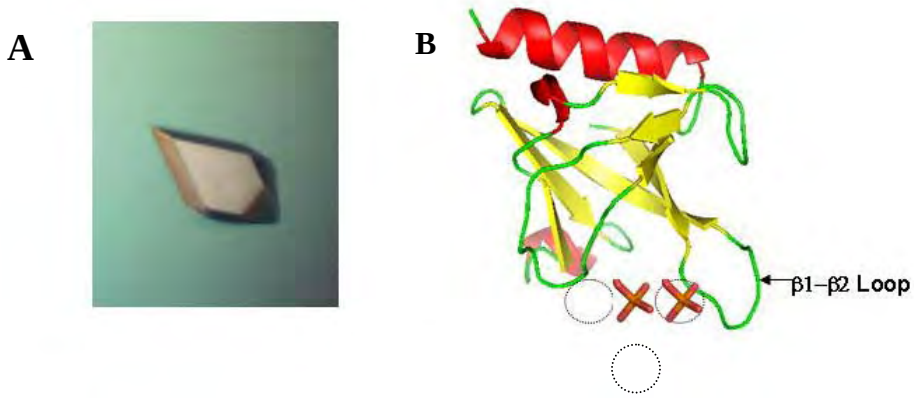


Figure 7 Protein crystal (A) and most refined current model of His-Osh1p PH (B)
8 mg/mL purified His-Osh1p PH crystallized using hanging drop method in 0.1M Na-acetate pH 4.6, 30% PEG-2000 ME, 0.2M$(NH_4)_2SO_4$, incubated at 21^0C for 1-2 weeks before crystal formed. Symmetry P 2121; Unit Cell 38.7 46.8 63.6 90 90 90; Resolution 2.2Å. A full data set was collected at home source and Molecular Replacement undertaken against DAPP1 PH (lacking side chains and with regions of divergence removed). Osh1p PH side chains were filled in on an $2F_0$-Fc electron density map and refined until R(free) reached ~0.32, after which refinements were made alongside composite omit maps. The coordinate pdb file is displayed in PyMol (B) with two adjascent phosphated in red and hatched circles where we are observing phosphate density.

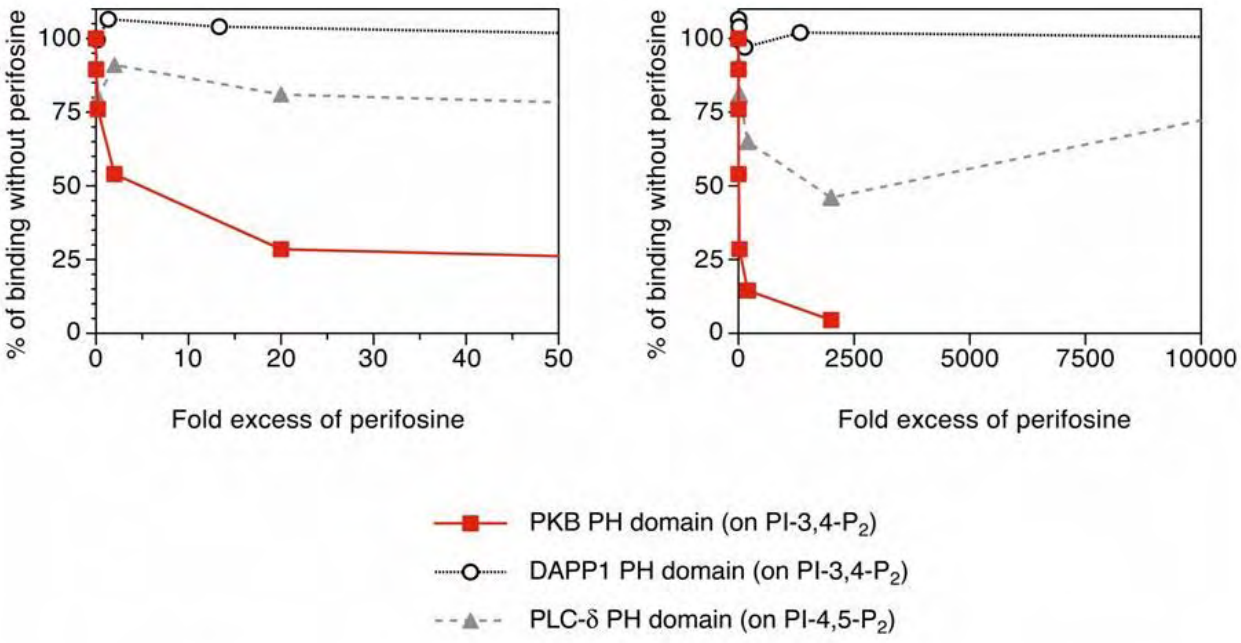


Fig.8 Perifosine selectively inhibits binding of PKB PH domain to PtdIns(3,4)P_2
SPR Binding Assay protocol to determine K_d as described in Yu *et al*, 2004. K_d concentrations used for perifosine competition experiments (PKB, 0.8 μM; DAPP1, 0.1 μM; PLCδ, 0.3 μM). PH domains incubated with perifosine for 30 min at RT before flowed over phosphoinositide-coated L1 chip. Increasing concentration of perifosine (x-axis) plotted versus RUs with perifosine addition, normalized against PH domain without perifosine (y-axis). DAPP1 PH domain is closest sequence-relative of PKB, and has essentially identical phosphoinositide binding specificity and affinity.

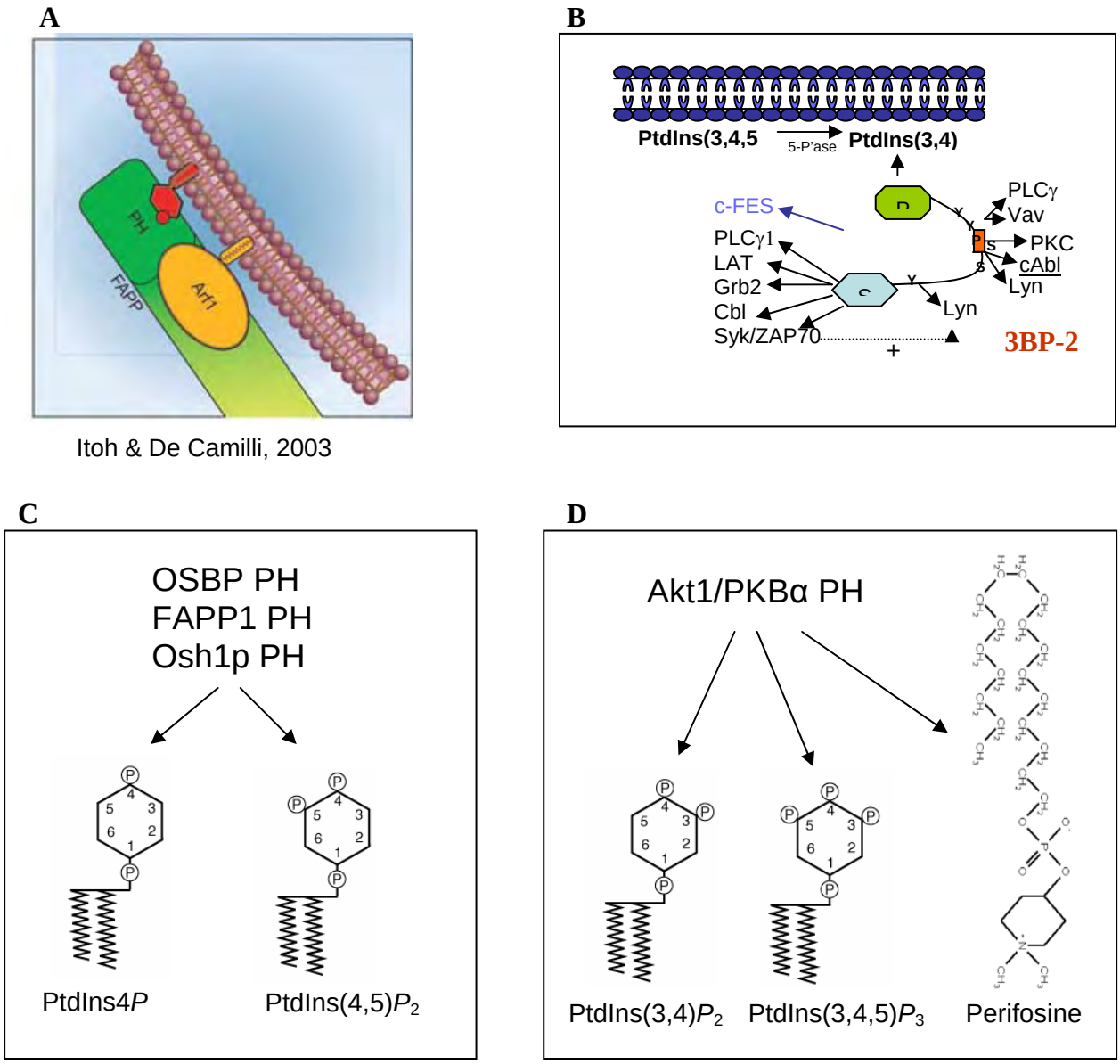


Figure 9 How do nonspecific and specific component interactions cooperate to drive selective membrane targeting?

Multipoint Contacts (protein-protein and protein-lipid interactions)

(A) FAPP1 PH targeting to Golgi via dual specificity for PtdIns4*P* and ARF1.

(B) SH3BP2 adaptor protein interacts with a variety of signaling protein via its SH2 and PR domains and targets to the plasma membrane with its affinity for PtdIns(3,4)P_2 upon receptor stimulation.

In both instances, relatively weak affinity, yet highly specific, protein-protein interactions appear to complement moderate affinity protein-phosphoinositide interactions.

Phosphoinositide Recognition : Not all PtdIns interactions are equal!

(A) Osh1p/OSBP/FAPP class of PH domains bind PtdIns4*P* and PtdIns(4,5)P_2 with comparable affinity *in vitro* , despite the former phosphoinositide having one less phosphate group.

(B) Akt1/PKBα PH demonstrates high affinity & specificity for 3'-phosphoinositides, but low affinity & specificity for the drug perifosine *in vitro.*

Appendix

Akt-1/Protein Kinase B-beta	Akt1/PKBα
Dual Adaptor for Phosphotyrosine and 3-Phosphoinositides 1	DAPP1
Dissociation constant (apparent)	K_d
Four Carbon (acyl chain)	C4-
Four-phosphoinositide Adaptor Protein-1	FAPP1
Glutathine S-Tranferase	GST
Green Fluorescent Protein	GFP
Histidine (tag)	His-
Isothermal Titration Calorimetry	ITC
myristoylated ADP-ribosylation Facor 1	myrARF1
OSBP homolog-1	Osh1p
Oxysterol-binding Protein-1	OSBP-1
Phosphate group	*P*
Phosphatidylcholine	PC
Phosphatidylinositol (4,5)-Bisphosphate	PtdIns(4,5)P_2
Phosphatidylinositol 3-kinase, 4-kinase	PI3K, PI4K
Phosphoinositide	PPIns
Phospholipase C-delta	PLCδ
Pleckstrin Homology	PH
Proline Rich	PR
SH3-binding Protein 2	SH3BP-2, 3BP2
Surface Plasmon Resonance (BIAcore)	SPR
Tritiated Inositol(1,4,5)-Trisphosphate	[^{3}H]-Ins(1,4,5)P_3